

**BINGE-EATING BEHAVIOR IN MICE: INFLUENCES OF RESTRICTION  
AND PALATABILITY IN A LIMITED ACCESS MODEL**

A Dissertation

by

KRISTINA DAVIS

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Psychology

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Approved by:

Chair of Committee,	Paul Wellman
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## **ABSTRACT**

Binge-Eating Behavior in Mice: Influences of Restriction and Palatability in a Limited  
Access Model.

(May 2008)

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Animal models of bingeing have typically used stress to induce bingeing. A recent model, limited-access to high-fat diet (HFD), has shown that caloric restriction and stress were not required to induce bingeing in rats. This study replicated this model in mice, explored the fat content within the model, and investigated locomotor activation associated with binge-eating. Adult mice were maintained on a restricted feeding (RF) schedule of 2 h/d of access to chow or ad lib access to chow, and then provided limited access to 45% HFD or 84% HFD for 30 min 3 d/ week for 6 total snack sessions. Circadian activity was monitored for RF animals offered 84% HFD, and after 6 snack sessions were complete, allowed continuous access to the 45% HFD or the 84% HFD for two weeks to explore rebound feeding. Bingeing, defined by increasing intakes across days, was reported for mice offered 45% HFD regardless of deprivation state (RF or ad lib), while mice offered 84% HFD only exhibited bingeing when they were restricted. Comparison of male and female mice maintained RF, offered 45% HFD snack, showed

that females had higher intake (kcal/g-bw) while ad lib fed mice exhibited no sex differences. Circadian recordings for female RF mice offered 84% HFD showed shifts in activity from the first hour of dark cycle to the hour preceding the snack and supported that offering the HFD produced alterations in food-associated arousal. During rebound, female RF mice given 84% HFD showed the highest intakes in week 1, and then exhibited a marked decline in week 2. The week 1 intake for RF animals were to regain lost body weight and that homeostatic-like intake in week 2 allowed normal body weight maintenance.

Results of this investigation support human data that females are more susceptible to binge-type eating disorder, shows that limited access to palatable foods for females under caloric restriction induces changes in circadian activity, and reveals that using mice in this model requires more investigation to optimize binge-behavior. Diet comparisons also suggest that homeostatic and reward mechanisms may have an additive effect on bingeing.

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## INTRODUCTION

According to the Diagnostics and Statistical Manual-IV [1], binge-eating disorders are classified by recurrent episodes where large quantities of highly palatable food are ingested within a short time period while experiencing a loss of control; these episodes may be followed by purging (e.g., bulimia-nervosa) or not (e.g., binge-eating disorder). Evidence exists in human eating disorder studies that palatability and restriction both influence ‘binge’ eating behavior [2, 3, 4]. The most recognized pathological bingeing disorder is bulimia-nervosa (BN) which consists of ‘binge’ episodes followed by compensatory measures (i.e., purging, excessive exercise, laxative abuse) to maintain a normal body weight (BW). In contrast, binge-eating disorder (BED) is characterized by increased BW and fat mass, because no purging of the high-calorie binge items occurs. A recent report posted on the National Institutes of Mental Health website states that all types of eating disorders are more prevalent in women than men [5]. This report gives the statistical prevalence for BN in the USA to be 1.5% of women and 0.5% of men, while BED is reported to affect 3.5% of women and 2.0% of men. Furthermore, approximately 10-15% of obese patients that present for treatment suffer from BED, making BED the most prevalent eating disorder.

The key and common features of BN and BED are both psychological (subjective) and overtly behavioral. Psychologically, bingeing is associated with

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This dissertation follows the style of Physiology & Behavior.

perceived restriction of palatable foods and/or calories [6] and negative affect in the form of body image dissatisfaction, guilt/shame about behavior, and/or lack of control over behavior [7, 8]. Binge-type disorders are often associated with high measures of impulsivity [9, 10] and neuroticism [11] and often occur co-morbidly with anxiety disorders lending support to the notion that negative affect may be part of the etiology and maintenance of these disorders. Behaviorally, bingeing is characterized by excessive intake of palatable food and ritualized pre- or post-binge behaviors (e.g., methods of attaining food items, methods of hiding the binge behavior, or purging/exercise). Bingeing shares common features with other types of addiction inasmuch as bingeing is associated with impulsivity, lack of behavioral control, and ritualized behaviors [9]. Therefore, the main problem with binge eating disorders, as with other addictions, is that they are fundamentally psychological in nature such that the physiological mechanisms that govern them are poorly understood.

Feeding, in general, is governed by a complex set of both physiological and psychological parameters such that determining which systems may be contributing to binge pathologies is incredibly difficult. In brief, the act of food intake is a multi-sensory process in that palatability/ taste [12], availability [13, 14, 15], and motivation [16] all influence the likelihood of feeding onset and duration. Furthermore, internal fluctuations in hormones (i.e., corticosterone, ghrelin, growth hormone, leptin, insulin, etc.) that signal peripheral-to-central information about the homeostatic needs (energy) of the body can act to modify sensory salience and create motivation for food intake [15, 17]. These hormonal fluctuations are responsive to the physiological needs associated

with changes in the body's energy stores, the circadian oscillations in energy expenditure, and food-associated learning [15].

Evidence suggests that people suffering from binge eating disorders may have metabolic or genetic alterations in functioning. For example, when obese bingers and non-bingers were shown food associated stimuli during fMRI, bingers showed right premotor area activation indicating that arousal of motor-planning areas of the brain may be a feature of binge-pathology or etiology [18]. Human clinical information about the physiological controls of binge-eating have implicated leptin [19], ghrelin [20], endocannabinoids [21], and serotonin [22] as potential modulators of bingeing inasmuch that binge-eaters tend to show alterations in many of the above hormones and neurochemicals when compared to non-bingers. However, given the co-morbidity of anxiety with binge-eating disorders, it is unclear how these complex neurochemical and hormonal pathways may individually contribute to binge pathology. Furthermore, polymorphisms in the genes for melanocortin 4 receptor (related to food intake), serotonin, and glutamic acid decarboxylase (catalyzes gamma-butyric acid formation) have all been associated with human binge-pathology according to the accrued information found on the NCBI Online Mendelian Inheritance in Man database [23]. This database also outlines that the genes for brain derived neurotrophic factor and catechol-o-methyltransferase (methylates monoamines) are linked to both anxiety disorders and binge-eating disorders. Together the genetic and neurochemical influences on binge-eating pathology are obviously complicated and difficult to separate from possible co-morbid mood disorders.

For humans, food restriction, either self-imposed [3] or parentally imposed [2] increases the likelihood of bingeing on restricted items when they are re-presented, and binge urges are directly related to the level of dietary restriction imposed [4]. Even in healthy women, not suffering from eating disorder, self-control for food reinforcement diminishes greatly as deprivation state increases [24], and after deprivation, palatability is positively correlated to the amount consumed [25]. Similarly, rats that are food restricted exhibit preferences for palatable, high-energy foods (e.g., sweets, fats) [26, 27]. Since a key feature of human binge pathology is restriction (or at least perceived restriction), most animal models of bingeing implement some method of restriction and/or access to palatable food in order to elicit binge-type behaviors.

As outlined above, binge-eating, by clinical definition, is not a homeostatic process and is likely to be governed by the complex interplay between psychology and physiology. Although the psychological constructs associated with binge-eating are nearly impossible to model, animal models of binge-type eating rely on behavioral mimicry induced by relevant manipulations (i.e., stress, palatable food, restriction). In brief, stress induced hyperphagia models rely on various forms of acute and chronic stressors that elicit marked eating behaviors in food satiated rodents [28, 29, 30, 31, 32; among others]. Restriction and refeeding regimens as well as restricted access paradigms rely typically on short-term access to palatable food and this induces increases in intake of the palatable food after multiple exposures to the restriction/refeeding cycle or restricted access period [28, 29, 32, 33, 34, 35]. These models will be discussed more fully below.

Historically, stress-induced hypophagia has been widely used as a model of bingeing and obesity-like behaviors. Rowland and Antelman [36] showed that adult female rats provided ad lib access to sweetened milk exhibited binge-like eating to a hand held milk bottle that was made available only during mild tail pinch sessions. With chronic tail-pinch stress (repeated pinches over 5 consecutive days), these animals became markedly obese and ceased to drink the palatable solution in their home cage. However, it has been shown that eating in response to tail-pinch is not dependent on the availability of palatable food in that standard chow intakes have been shown to increase after tail-pinch stress as well [37]. This evidence for stress-induced eating suggests that eating may serve as a potential ‘coping’ or recuperative response to physical stressors.

Furthermore, stress-induced eating appears to have carry-over effects in that prior experience with stress-induced bingeing predisposes animals to eat in response to new stressors. For example, Wilson and Cantor [31] found that tail-pinch induced almost a 600% increase in wet mash (chow + water) consumption compared to baseline mash diet consumption and that a history of tail pinch (6 sessions) carried forward such that those animals ate more mash diet in response to a novel, loud noise stressor. This suggests that previous experience with stress-induced binge-eating influences future behavioral responses to palatable food either through learning or permanent physiological changes in the organism after such experiences.

Exposure to stress early in development has also been shown to influence adult binge-eating behaviors. When rat pups were exposed pre-weaning to daily 15 min handling separation, male and female animals ate more of a palatable snack (graham

wafers) after weaning than did non-handled controls [32]. Furthermore, female animals, but not males, that were exposed to repeated 3h maternal separation before weaning ate more of the palatable snack after 4 days of exposure to the snack than non-separated control animals. This evidence suggests that separation stress during development can influence binge-like eating behavior of a palatable food after maturation.

Models of restriction/refeeding (R/R) have been used often because they more closely mimic the self-inflicted cycles that human dieters and bingers often experience. R/R models employ cycles of restricted caloric intake countered by ad lib phases and typically use some form of stressor to induce bingeing after repeated exposure to R/R cycles. For example, Hagan [36] exposed female mice to R/R cycles consisting of a 4 day restriction period with either a 75% or a 50% of normal intake followed by refeeding period of 2 days ad lib feeding. After 6 iterations of this cycling (84 days total), these animals, including a non R/R control group, were then allowed 30 days of ad lib chow feeding. At the end of the 30 day ad lib period, a history of R/R produced increased feeding in response to a 24h deprivation period, and this effect was increased when palatable food (cookies) was offered in addition to chow after the 24 h deprivation period. In this study, the 24 h deprivation acts as a stressor and shows that stress-induced bingeing is entrainable inasmuch as animals that had a history of R/R cycling were more sensitive to this stressor. Additionally, female rats exposed to restriction (66% of normal intake) and refeeding cycles, which included a foot shock stressor during the restriction period, expressed binge-type eating only when the diet offered was palatable. Animals exposed to either R/R or foot shock stress alone did not show this type of bingeing [29].

This binge-eating of a palatable diet in response to foot-shock stress and R/R cycles has been replicated by others [30]. However, further work by Hagan [28] showed that R/R with foot shock stress during the restriction period produced a significant increase in palatable diet intake, and that standard chow intakes were also increased after this treatment but only if a taste of the palatable diet was provided to chow-eaters. In summary, the work of Hagan and colleagues shows that R/R cycles produce lasting effects such that bingeing in response to stressors is increased and that the addition of a stressor to R/R cycling can induce binge-like episodes during re-feeding. This methodology incorporates some key features of human pathology in that negative affect (stress) and restriction cycles are both used, making it a valid model of binge-eating behavior.

When animals are trained to eat all of their daily calories within a discrete time period they will exhibit avid feeding when ad lib access is returned. Inoue [33] maintained female rats on either 2 weeks of 2h/d access to chow or a single 22 h deprivation period and then allowed ad lib access to chow for 24 h. During the 24 h re-feeding period, animals kept on the scheduled feeding ate significantly more than did the animals treated with the one 22h deprivation period. Furthermore, the increased food intake in response to the limited access to chow each day appeared to alter the stress-eating response in a subset of animals were subjected to space-restriction stress during the re-feeding period. Animals subjected to a small chamber during the 24 h re-feeding period, after being schedule-fed 2h/d, exhibited an exacerbated re-feeding response. Although the re-feeding after 2 h/d access may not readily be classed as 'binge' because

it supports a compensatory behavior to regain lost BW, the fact that this type of restricted schedule creates an enhanced 'binge' in response to stress does model some portion of human pathological behaviors. It might be argued that a 2 h/d schedule closely mimics human self-restriction patterns that are seen in binge-pathologies and that this methodology then presents a valid model of stress-induced bingeing for restricted eaters.

The limited-access 'snack' paradigm models human binge-eating in that it limits the access to a palatable food option without restricting standard chow. Therefore, this model most closely reproduces the type of restriction that is seen in BN and BED where normal to increased BW is maintained but restriction of palatable foods is imposed. Corwin's [34] first publication of this model reports that male rats, which were provided ad lib chow throughout, exhibited increased intake of vegetable shortening when access to the palatable shortening was only allowed for 2 h per day on 3 days of each week. Specifically, male rats were provided 2 h access to vegetable shortening on either each day of the week (low-restriction group) or on Mondays, Wednesdays, and Fridays only (high-restriction group). Results indicated that limited access every day each week did not result in increasing intake over time but that animals on high-restriction regimen increased their intakes compared to the low restriction group and control animals after 4 access periods. By the end of 2 weeks of access to vegetable shortening, animals in the high-restriction group ate 51% of their daily caloric intake during the 2 h access period. However, exposure to either limited-access regimen did not result in increases in BW compared to control animals which never had access to



vegetable shortening. This limited-access model was later replicated in female rats [35]. Comparison of male and female animals in these published limited-access studies shows that after 2 weeks of limited access (3 days/ week) females ate more in the 2 h snack period than did male animals with the same treatment lending support to the idea that females are more susceptible to binge-eating than males.

In the limited access ‘snack’ model, the 3 day per week schedule was required to induce the binge-effect. This presents a possible shortcoming of the model in that because each intervening day constitutes a day in which the animals are subjected to caloric/fat deprivation such that intake is increased to compensate during the next access period. This issue was addressed by Corwin [39] where the model was replicated as described above, except a group was added that received 3 days per week access on a variable schedule (not regularly Mondays, Wednesdays, and Fridays). The addition of this group provided a solution to the perceived deprivation in that animals in this group had some access periods that occurred on successive days (eliminating any intervening deprivation day). The schedule was such that these animals received successive access days during the last 2 access periods because this is the time in which the binge-effect was most likely to appear (after 2 weeks); results support the validity of this method as an accurate model of binge behavior. Animals offered 3 d/ week access to vegetable shortening ate significantly more shortening than controls or animals offered 7 d/week access, and the effect was still evident for the group in which deprivation days were limited at the end of the experiment. The binge-effect produced by limited access to vegetable shortening appears to be fairly robust and applicable to both genders while

being independent of deprivation state for the animal. This model truly does appear to be a learning- or experience-induced binge-effect.

The limited-access binge-effect has also been shown to occur when the item for consumption is alcohol [40] in that rats trained to lever press for 10% ethanol consumed more in a 30 min period if they were only allowed ethanol reward for one 30 min access period per day compared to animals offered from 2 to 16 access periods per day. This suggests that the binge-effect may be linked to reward properties of the restricted item and therefore potentially an addictive-like behavior that is not overtly controlled by energy homeostasis. However, these behaviors may also be indicative of human consumption patterns that are not pathological [40, 41, 42]. Energy restriction is not necessary to produce this ‘binge-type’ eating behavior in rats [34, 35, 39]; however, in human binge-eating disorders; restriction (or at least perceived restriction) is an important aspect of the behavioral profile [3].

Binge-eating and overeating of palatable foods in general, is likely governed by the same neurochemical and neuroanatomical mechanisms that control reward to other types of stimuli. Furthermore, evidence suggests that the dopamine pathways that mediate restriction-induced bingeing (energetically driven) and food reward based on palatability can be dissociated [for review, see 43]. Similar dissociations of need-based intake and ‘want’-type intake have been proposed for salt palatability as well [for review, see 12]. An investigation into this concept of dissociation within the R/R binge models yielded evidence that both palatability and a history of restriction influenced reward systems and affective behaviors [44]. Specifically, when female rats were

subjected to intermittent, but not daily, access to palatable food (cookies), they exhibited decreased dopamine levels in the reward-relevant anterior hypothalamus. This altered dopamine was not dependent on a history of restriction suggesting that exposure to intermittent access to palatable diet induces changes in reward and feeding relevant pathways. However, animals exposed to R/R cycles and intermittent access to palatable food exhibited decreased serotonin and dopamine levels in the medial prefrontal cortex, increased depression-like behaviors (as measured by a forced swim test), and increased novelty avoidance (as a measure of anxiety). Animals provided access to the palatable diet alone or R/R cycles alone did not express these alterations in affect-associated measures. This evidence suggests that the combination of palatable diet access and restriction creates alterations in affect and reward-relevant neurochemistry lending support for the altered hormonal and neurochemical issues associated with eating disorders.

Given the evidence above for alterations in neurochemistry [44] induced by restriction and access to palatable diets, it is not surprising that both of these manipulations have been shown to create alterations in other behaviors, especially activity patterns. Rodents are nocturnal and therefore eat most of their daily calories and exhibit more locomotor activity during the dark cycle. Many researchers purport the existence of a food-entrainable oscillator, separate from the suprachiasmatic nucleus, that governs the circadian control of food intake and locomotor activity (arousal) associated with food intake [for review, see 45]. Restriction of food intake or scheduled feeding does appear to alter activity patterns in rodents. For example, a 2 day food

deprivation period altered the expression of circadian related genes (e.g., *Dec1* and *Per1*) [46]. Also, when rodents were only allowed access to food for 2 h per day, they exhibited increases in activity in anticipation of food access and after the conclusion of the feeding period [47]. This anticipatory activity persisted after ad lib access had been returned. This result has been replicated in that Lax [48] and Yokoyama [49] both reported similar changes in activity after subjecting rats to a 4 h and 3 h per day feeding period, respectively.

In addition to restriction regimen, exposure to high-fat diet (HFD) also appears to alter the circadian activity patterns of rodents. When animals were offered a palatable diet during the light cycle, but otherwise not restricted, they shifted their food intake patterns such that 80% of their daily calories were consumed during the light cycle, and they increased their activity during the light cycle [50]. In contrast, Bartol-Munier [51] reported decreases in overall 24 h activity levels during exposures to HFD. This evidence supports the notion that exposure to HFD can produce variations in circadian activity rhythmicity, with some variability in findings. However, Panskepp [50] allowed free access to the HFD during every light cycle (but not at night) and Bartol-Munier [51] provided daily continuous access, so the discrepancy in these two studies may be related to the variations in access to the HFD. Therefore it is plausible that limitations on access to HFD, as seen in limited-access models, may produce alterations in activity patterns that are associated with the access to the palatable diet and that restriction of caloric intake, as seen in R/R, may also produce alterations in arousal and circadian activity levels. Elucidating potential alterations in circadian activity levels associated with

caloric restriction and limited access to palatable foods may be important as an indicator of food-associated arousal in human binge-eating pathologies. As mentioned above, human premotor areas are activated by food stimuli in binge-eaters [18], and this may be one of the first clues that motor arousal may be an important aspect of binge-eating. The animal literature indicates that circadian cues associated with feeding can alter locomotor activation; therefore, it is plausible that human binge-eating may be influenced by similar circadian cues.

Exposure to restriction and/or bingeing may also produce changes in subsequent ad lib feeding behavior. Homeostatic regulation can explain rebound feeding that occurs after replacement of ad lib access where intakes are typically high to recoup lost weight. However, long-term exposure to ‘diet’-type conditions, i.e., 2 h access to food per day, caused female rats to have higher rebound food intakes than animals exposed to an acute 22 h deprivation period even though no differences existed in BW between groups [33]. This suggests that prolonged food-restriction alters rebound feeding response that cannot be entirely explained by homeostatic mechanisms. Furthermore, when a subset of these female rats were subjected to space-restriction stress (i.e., small chamber that hindered mobility) for the duration of the 24 h rebound period, the hyperphagia after exposure to the 2 h per day scheduled feeding was further increased above animals subjected to only the acute deprivation. This suggests that, in addition to increases in overall rebound feeding, the ‘diet’-like scheduled-feeding restriction induces alterations in hyperphagic response to stress. A history of restriction may also cause long-term alterations in stress-induced eating. Hagan [38] reported that animals subjected to 12 weeks of R/R cycling,

allowed to free access to chow for 30 days, and then subjected to a 24 h deprivation period showed increased hyperphagia compared to animals with no history of restriction. Additionally, animals that were allowed access to both chow and a palatable option (cookies) during refeeding segments of the R/R cycles exhibited increased intake of cookies after a 24 h deprivation which occurred 30 days after R/R cycles had ended. Animals provided cookies, but not calorically restricted, consumed similar amounts of cookies to chow-access only groups after the 24 h deprivation challenge. In summary, 30 days after R/R had ended, hyperphagia in response to acute deprivation stress was increased by previous restriction, especially when a palatable diet was offered. However, when animals subjected to 12 weeks of R/R and then 30 days of ad lib feeding were allowed access to cookies and chow without an acute deprivation-stress, previous restriction did not affect the level of intake but a previous access to the cookies did increase intake. In short, both a history of restriction and a history of access to palatable foods appear to create long-term changes in food intake of palatable diets and stress-induced hyperphagia, increasing the magnitude and likelihood of binge-eating.

Animal models exist in rats for ‘binge-type’ eating of palatable foods and these models are likely influenced by factors such as the deprivation state of the animal, a exposure to palatable foods, and the potential circadian shifts that may occur during scheduled feeding. The focus of this investigation was fourfold: 1) to replicate the rat limited access model in mice, 2) to determine how fat content (palatability) and restriction (calories) may influence bingeing within a limited-access model for mice offered 45% or 84% HFD snacks, 3) to explore the alterations in circadian rhythms that

may occur for restricted and unrestricted mice provided HFD snacks within the limited access paradigm, and 4) to examine rebound feeding after limited access to a palatable diet.

To date, no research is published evidencing success of a limited-access model in mice, and since mice are the mainstay of genetic research, establishing the success of this model in mice will be important for further research on the study of gene-regulated metabolic factors in BN and BED. As introduced above, human genetic research has linked multiple genes to binge-eating behavior, thus, a valid animal model is needed to fully investigate the roles of these genetic loci in binge-behavior. The first aim of this investigation was to explore high-fat diet (HFD) consumption in a limited-access paradigm in mice that were both ad lib- fed chow and mice that were maintained on a restricted feeding (RF) schedule of 2 h access to chow/ d. Because human binge-pathologies are often accompanied by restriction, this study sought to compare the unrestricted limited access model [34] with the same model that includes a ‘dieting’ component. Because this model is typically implemented with a vegetable shortening HFD option, the current research also sought to determine if this model would be successful with a 45% HFD option that contains complete nutrition (Experiment 1) versus an 84% HFD (Experiment 2a) that is more similar to the pure-fat vegetable shortening used by Corwin and colleagues [34, 39].

In order to investigate how the limited-access period might alter locomotion patterns for animals maintained on both RF and ad lib feeding schedules, animals run in the limited-access model were monitored for circadian activity patterns during the

limited-access period (Experiment 2b). After completion of 2 weeks of limited access to HFD, a subset of animals were provided ad lib HFD with either the 45% or 84% fat by calories in order to explore rebound weight gain and food intake in mice after exposure to limited-access paradigm (Experiment 2c). [See Table 1 for a summary of treatments.] For this rebound portion of the experiment, half of the animals were provided a familiar HFD (previously used during limited-access) and half were presented a novel HFD (different from the one seen in snacking) in order to determine how past experiences might influence rebound feeding behavior.

Table 1  
Summary of treatment conditions

	Sex	Diet schedule	Snacking	Rebound
Exp1	Male	ad lib	45% HFd	
	Male	RF	45% HFD	
	Male	RF	chow	
	Female	ad lib	45% HFD	
	Female	RF	45% HFD	
	Female	RF	chow	
Exp2	Female	RF	84% HFD	45% HFD
	Female	RF	84% HFD	84% HFD
	Female	ad lib	84% HFD	45% HFD
	Female	ad lib	84% HFD	84% HFD



## METHOD

### *Animals*

All animals were adult F2\_129SvEvBrd x C57Bl6J albino mice (Lexicon Genetics, The Woodlands, TX). Mice were individually housed at 10 weeks of age in standard polycarbonate mouse cages (29.2 x 19.1 x 12.7 cm) with water available ad lib in a temperature and humidity climate controlled procedural room maintained on 12:12 light: dark schedule, lights off at 07:00 p.m. CST. All mice were allowed to habituate to individual housing in the procedure room for 2 weeks prior to the onset of testing procedures. In experiments using female animals, estrous phase was not measured or accounted for other than by ensuring adequate acclimation time to housing conditions. All procedures were approved by Lexicon Pharmaceutical's IACUC and adhered to the NIH Guide for the Care and Use of Laboratory Animals [52].

### *Diets*

All diets were obtained from Purina Mills/TestDiet (Richmond, IN). During habituation to the procedural room, mice received ad lib standard rodent chow (TestDiet product# 5010). At the onset of restriction procedures, standard rodent chow was presented in 500 mg pellets (TestDiet product# 5010) to enable faster and more accurate estimation of food intake. For HFD procedures either a 45% calories-from-fat (TestDiet product# 58V8) or an 84% calories-from-fat (TestDiet product# 5TJQ) diet was offered. Table 2 contains full nutritional information for all diets. United States Department of Agriculture research cites that the average American obtains approximately 35% of their daily calories from fat [53].

Table 2  
Nutritional content for experimental diets

Diet Type	Test Diet#	kcal/g	% calories obtained from:		
			fat	carbohydrate	protein
Chow	5010	3.41	27.5	13	59
45% HFD	58V8	4.65	45.7	35.5	18.3
84% HFD	5TJQ	6.15	83.9	0	16.1

### *Restricted Feeding (RF) Procedures*

Animals were individually housed in the procedure room for a minimum of 10 days with water and standard rodent chow ad lib prior to the onset of restriction. Food was removed on the Sunday before restriction procedures began; water was available ad lib throughout the experiment.

On day 1 of restriction feeding (RF), mice were offered 500 mg chow pellets for 4 h starting at 09:30 a.m. CST. Over days 2 through 4, the duration of the feeding period was reduced from 4 h to 2 h. This reduction in feeding time was used to reduce the rate of weight loss and allow the mice to learn to eat their entire daily allotment within the limited time period. Subsequently, mice were offered access to 500 mg chow pellets from 09:30 – 11:30 a.m. each weekday, and were allowed unrestricted access to their allotments on weekend days. At the end of the feeding period (11:30 a.m.), food pellets and spillage that were remaining were recorded. Spillage for these diets was negligible. After training in the RF procedures (5 d), most animals ate the entire allotment before the 2 h feeding period was complete.

On average, mice consumed approximately 2 - 4 g of chow each day and maintained 85% – 90% of free-feeding body weight (BW). Limited-access procedures were not implemented until all animals showed evidence of stable BW in that they

maintained 85% - 90% of free feeding BW for 5 consecutive days. BW was recorded 5 d/ wk just prior to the addition of the daily food allotment (09:30 a.m.). For animals that were not restricted, BW was recorded at 09:30 a.m. and any additional food or water was added at this time such that animals received identical cage and handling manipulations regardless of feeding schedule (ad lib versus RF).

#### *Snacking Procedures (Limited-Access)*

In order to avoid neophobia upon presentation of the HFD, 4 days preceding the onset of snacking procedures all animals that were to receive HFD during snacks were offered a one-time exposure to 1 g of the appropriate HFD. Snacks were offered at 2:30 p.m., and ‘snack’ food remained available for 30 min. For animals maintained on ad lib chow, chow was removed just prior to administration of the snack diet, such that for all animals, the snack diet was the only available food for the 30 min period. At the end of the 30 min snack period, any surplus snack diet was removed from the cage and weighed, and for ad lib animals, chow was replaced.

Upon presentation of the ‘snack’, latency to begin eating was recorded by a single experimenter with a stopwatch for mice in the RF condition. Latency to begin eating, recorded to the nearest 1 s, was defined as approach and 10 s of continuous intake. The 10 s criterion was used to account for the normal rodent behavior which often consists of multiple approaches and ‘tastes’ (i.e., exploration-like behaviors) that may occur prior to meal onset. Latency was not recorded for ad lib fed animals because of time restrictions during the test. For ad lib animals, pilot work showed that latency to

begin eating was typically greater than 5 min, and manual recording of this measure for multiple animals produced interference with the 30 min testing schedule.

*Procedures: Experiment 1*

Two groups of animals were maintained on either ad lib standard chow pellets or placed on the RF regimen outlined above. The RF animals (Male, n = 24; Female, n = 18) were further subdivided such that half the animals were assigned to receive 500 mg chow pellets during snacking procedures, while the other half received 45%HFD during snacks. All animals in the ad lib group (Male, n = 18; Female, n = 14) received 45% HFD during snacks. See Table 1 for complete summary of treatment conditions.

After separation and RF training days as outlined above (and equivalent individual housing for ad lib groups), all animals were offered a snack at 2:30 p.m. RF groups received their normal feeding period in the morning (09:30 to 11:30 a.m.), and were presented with approximately 20 kcals of either 45%HFD or chow pellets for 30 min. The ad lib group also received the afternoon snack (2:30 p.m.) in which chow was removed from the cage and 45%HFD was provided. At the end of 30 min, any surplus snack diet was removed and weighed to the nearest 0.1 g. Chow was replaced for the ad lib group.

All animals received 6 snacks offered on Monday, Wednesday, and Friday for 2 weeks. Daily food intake, snack intake, and BW were recorded throughout.

*Procedures: Experiment 2*

HFD snacking. In order to examine influence of higher fat content/palatability on mouse snacking/bingeing behavior in RF versus ad lib animals, Experiment 2 consisted

of naïve female mice maintained on RF schedule ( $n = 20$ ) and on an ad lib ( $n = 14$ ) feeding schedule. All animals received an 84% HFD snack offered in the afternoon instead of the 45% HFD offered in Experiment 1. Females only were used due to equipment space constraints and because binge-eating disorders are historically more clinically relevant to female populations and have been more often studied in female rodents. All other procedures were the same as Experiment 1. Data Analysis was the same as in Experiment 1. (See Table 3 for summary of treatment conditions.)

Circadian recording. Circadian measurement (daily 23 h locomotor activity measure) was conducted using a photo beam array (San Diego Instruments, San Diego, CA) designed for use around the standard home cage which records beam breaks continuously using Flex-Field software (San Diego Instruments, San Diego, CA). Circadian equipment continuously recorded horizontal activity for all chambers for 23 h/d beginning at the onset of the 9:30 a.m. feeding period and ending approximately 15 min before the onset of the morning feeding period for RF animals. The period during which circadian recording was suspended allowed for data extraction and resetting of circadian equipment such that circadian recording began again at the onset of the feeding period. Circadian recording was not interrupted during afternoon snacks.

Circadian data was collated such that the sum of the activity counts during specific 1 h increments for each animal for each day was used in analysis. Repeated Measures ANOVA was utilized to explore the changes in activity that occurred over the 2 week snacking period for each of the 1 h increments. Because scheduled feeding and snacking procedures were expected to create shifts in activity such that predictive

activity should increase as the RF feeding period approached for animals trained to eat during this period, the activity sums for the hour preceding the RF feeding period (prior to experimenter entry into the room) as well as the second hour of the feeding period (after the experimenter left the room) were collated. Additionally, after repetitive snacking, predictive activity should present during the hour preceding the afternoon 84% HFD snack and potentially remain high for the hour after the afternoon snack. The hour after the onset of the dark cycle is typically the highest activity level for rodents. As a comparison measure, this hour (7:00 – 8:00 p.m.) was summed such that baseline circadian activity differences could be determined throughout the course of the ‘snack’ schedule.

Rebound feeding. Upon completion of all HFD snacking and circadian procedures, animals were shifted to ad lib access to HFD. Specifically, the animals that were offered 84% HFD snacks were provided ad lib access to HFD after snacking procedures are completed. Both RF animals and animals fed continuously ad lib throughout snacking were each divided into 2 groups, one receiving 45% HFD (RF: n = 7, AL: n = 7) and the other receiving 84% HFD (RF: n = 10, AL: n = 10) during the 2 week rebound period. See Table 1 for a complete summary of the treatments that each group of animals received. Food intake and BW were recorded daily throughout the 2 week rebound period.

### *Statistical Analysis*

For all snacking experiments and the rebound feeding, Repeated Measures Analysis of Variance (ANOVA) was used to examine measures of body weight (BW),

snack intake, and latency. During the 30 min snack period, the groups receiving HFD (either 45% or 84%) had maximum intakes of greater than 3 g by day 6 of snacking procedures; groups receiving chow snacks consumed maximum weights of approximately 2 g in 30 minutes. Therefore, intake of animals receiving chow was not limited by the increased volume needed to consume equivalent calories to HFD. Because no ceiling effect was evident for chow groups in terms of volume of intake, all further analyses were conducted using kilocalories (kcal). Food intake during snacks was converted from the observable g measure to kcal in order to make diet comparisons independent of the caloric density (see Table 2 for conversion factor for each diet). Furthermore, because restricted fed animals weighed less than ad lib fed animals and sex differences in BW were observed (see Table 3), the calculated variable of intake per g of BW (kcal/ g-bw) was used in analyses because this normalized intakes in light of differences in BWs produced by the different treatment conditions.

Circadian data was collated such that the sum of activity counts that were recorded during 1 h increments for each animal for each day was used in analyses. Increments of interest were the hour preceding the RF feeding period (before experimenter entered the room), the 2<sup>nd</sup> hour of the RF feeding period (after the experimenter left the room), the hour preceding the afternoon 84% HFD snack, the hour after the afternoon snack, and the hour after dark onset (7:00 p.m.). Repeated Measures ANOVA was utilized to explore the changes in activity that occurred over 2 weeks for each of the 1 h increments listed above.

Where significant interaction effects were present, post hoc analyses were performed using the Tukey HSD (t-tests). Planned comparisons, Student's t-tests, were performed within each group for day 1 versus day 6 of snack tests because this model defines bingeing as the increase in intake after multiple exposures. The estimate of effect size is reported as partial eta squared ( $\eta_p^2$ ) for all ANOVA results. All statistics were computed with Statistica (v.8) for Windows (Statsoft Inc., Tulsa, Oklahoma, USA).



## RESULTS

### *Experiment 1*

Separate two-way Repeated Measures ANOVAs were performed for each measure (BW, latency, snack intake) for animals offered the 45% HFD as a snack with day x snack diet (chow or 45% HFD) for animals that were restricted and with restriction schedule (RF or ad lib) as the between subjects factor and day as the within subjects factor.

45% HFD versus chow snacks (RF). BW for RF females offered the 45% HFD snacks increased over multiple exposure to snacks,  $F(5, 35) = 14.16$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.67$ , while females maintained on RF and offered chow snacks exhibited no such increases (see Table 3). Males had a similar pattern of BW changes over days,  $F(5, 50) = 3.57$ ,  $p = 0.008$ ,  $\eta_p^2 = 0.26$ , with RF animals offered 45% HFD having increases in BW across days. Males had significantly higher BW compared to females on all snack days when offered the 45% HFD,  $F(1, 50) = 72.60$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.59$ , or the chow snack,  $F(1, 57) = 66.22$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.54$ . [See Table 3 for complete listing of BW means (+/- sem).]

Analysis of latency (see Fig. 1) produced no significant differences for females and a significant day x diet interaction for males,  $F(5, 50) = 2.68$ ,  $p = 0.03$ .  $\eta_p^2 = 0.21$ . Post hoc analysis of male data showed that latency did not change across HFD snack days (repeated snacks) for animals offered either chow or 45% HFD during snack. As seen in Fig. 1, the interaction effect appears to be driven by a significant increase in

Table 3

Body weight averages for each condition for each snack day (+/- SEM)

	Restricted Fed Animals				Ad lib Fed Animals			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	Chow snacks		45% HF snacks		45% HF snacks		84% HF snacks	
	Female	Male	Female	Male	Female	Female	Male	Female
HFD1	23.1 +/- 0.44	28.9 +/- 0.58	22.4 +/- 0.46	28.7 +/- 0.94	23.0 +/- 0.57	36.3 +/- 1.61 *	38.4 +/- 1.91 *	21.8 +/- 0.63
HFD2	23.9 +/- 0.54	29.7 +/- 0.86	23.5 +/- 0.41	30.0 +/- 0.79	23.6 +/- 0.66	36.5 +/- 1.62 *	38.5 +/- 1.95 *	22.0 +/- 0.64
HFD3	23.2 +/- 0.46	28.6 +/- 0.81	23.2 +/- .79	28.8 +/- 0.84	22.2 +/- 0.66	36.4 +/- 1.60 *	38.4 +/- 1.95 *	22.1 +/- 0.63
HFD4	23.1 +/- .046	28.3 +/- 0.70	23.2 +/- 0.70	28.6 +/- 0.88	23.5 +/- 0.73	36.4 +/- 1.57 *	38.3 +/- 1.98 *	22.3 +/- 0.62
HFD5	24.1 +/- 0.40	30.12 +/- 0.82 †	24.1 +/- 0.56 †	31.1 +/- 0.84 †	23.5 +/- 0.70	36.6 +/- 1.57 *	38.4 +/- 1.96 *	22.3 +/- 0.62
HFD6	23.9 +/- 0.56	28.4 +/- 0.75	23.5 +/- 0.66	29.4 +/- 0.92	22.7 +/- 0.68 †	<sup>a</sup>	<sup>a</sup>	24.4 +/- 0.44 †

\* p &lt; 0.01 compared to corresponding RF animals

† p &lt; 0.05 compared to HFD1

<sup>a</sup> data not recorded due to human error

latency on snack day 3 for males offered chow snacks. Because this difference in latencies did not persist, data recordings were checked for errors or outliers that may have influenced the mean for this group on this day. No statistical outliers were found ( $\pm 2$  SD from mean) therefore this anomaly cannot be explained.

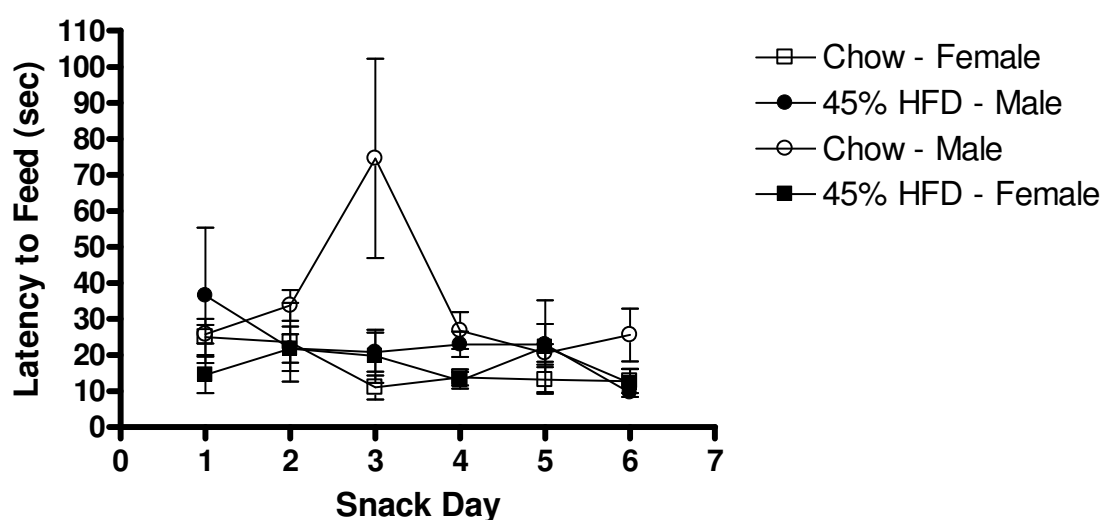


Fig. 1. Latency to Feed in 45% HFD Snacks. Latency to begin eating after snack presentation for animals maintained on a restricted feeding schedule (2 h access each day) that were offered either a CHOW snack or a 45% HFD snack for 30 min. Snack days occurred Mondays, Wednesdays, and Fridays afternoons.

Intake analyses were performed on the calculated measure of kcals/ g-bw for each animal across snack days. Due to human error, BW measures were not recorded for day 6 of snacking, therefore, for day 6 the average BW for each animal was used in calculation of intake scores. As seen in Fig. 2a, RF males offered the 45% HFD snack

consumed significantly more kcals/ g-bw than RF males offered the chow snack on all days except day 1 and day 5, day by diet interaction:  $F(5, 50) = 10.37$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.51$ . As depicted in Fig. 2b, RF females offered 45% HFD snacks consumed more kcals/ g-bw than RF females offered chow snacks 2 snack exposures, day by diet interaction:  $F(5, 35) = 7.33$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.51$ . Post hoc analyses for this interaction effect revealed a significant increase in intake for RF females offered 45% HFD snacks, while RF females offered chow snacks exhibited decreased intake between snack day 1 and snack day 6.

Comparison of sex differences in intake revealed that both males and females offered 45% HFD snacks while maintained on RF exhibited increased intakes across days,  $F(5, 45) = 17.62$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.66$ , and that females ate more kcals/ g-bw than males,  $F(5, 45) = 3.73$ ,  $p = 0.01$ ,  $\eta_p^2 = 0.29$ . RF females offered chow snacks consumed more kcals/ g-bw than RF males offered chow snacks,  $F(1, 8) = 14.08$ ,  $p = 0.005$ ,  $\eta_p^2 = 0.64$ . For both sexes, RF animals receiving chow snacks consumed less across days of snacking,  $F(5, 40) = 5.48$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.40$ . Additionally, a significant day by sex interaction was revealed,  $F(5, 40) = 2.63$ ,  $p = 0.04$ ,  $\eta_p^2 = 0.25$ , in that females offered chow snacks exhibited a more marked decrease in intakes over snack days than did males.

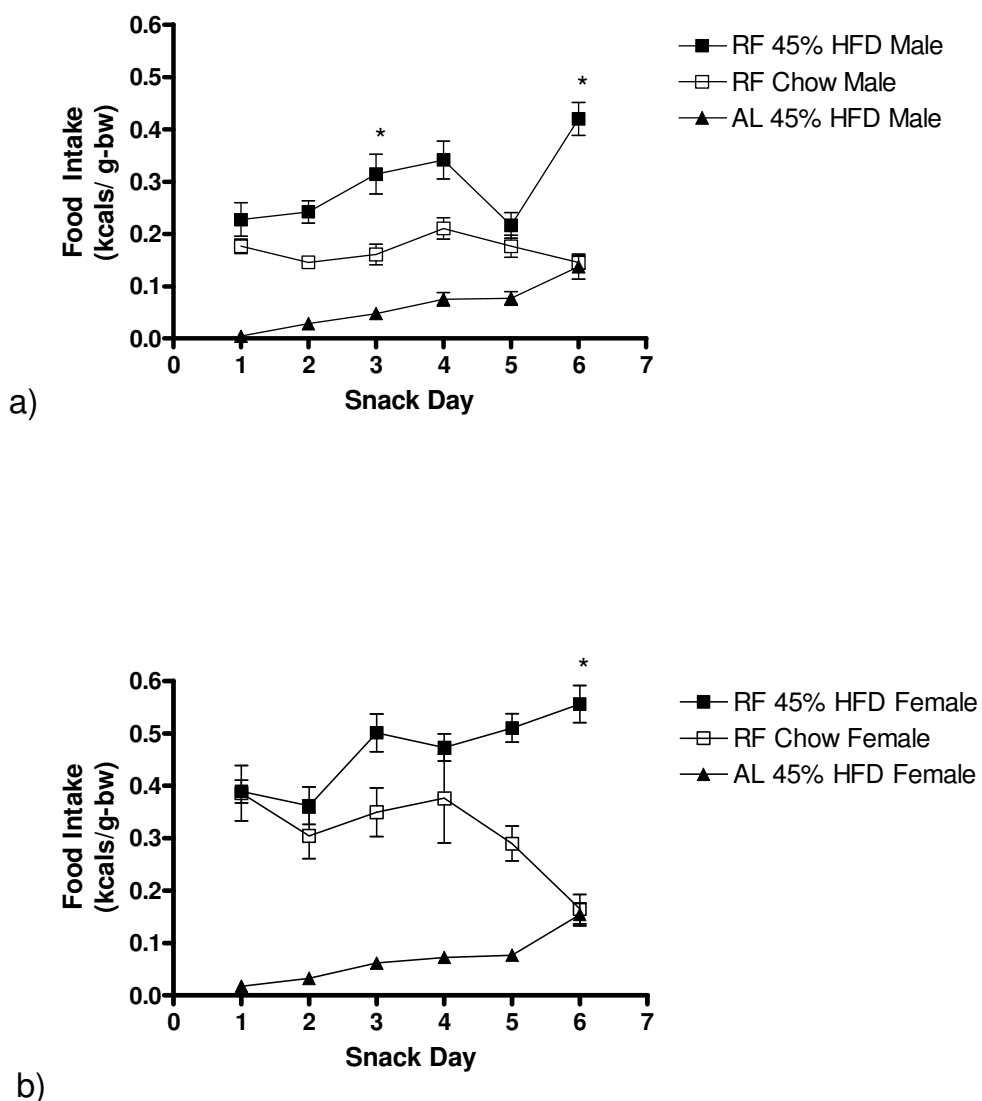


Fig. 2. Snack Intake for 45% HFD Snacks. Intake in kcal/g-BW for mice maintained on both a restricted feeding schedule and ad lib access that were offered either chow or 45% HFD during a 30 min snack. Snack days occurred Mondays, Wednesdays, and Fridays afternoons. Snack day 6 is not represented here due to missing data because of lost BW recordings such that kcal/g-bw could not be calculated. RF = restricted fed animals (2 h access to chow /d); AL = ad lib chow; \*  $p < 0.05$ . a) Males b) Females

RF versus ad lib fed animals (45% HFD snacks). Analysis BW (Table 3) for male animals offered 45% HFD snacks yielded a day by feeding schedule interaction,  $F(4, 152) = 15.79, p < 0.001, \eta_p^2 = 0.29$ . Post hoc tests revealed that RF animals had a significant increase in BW between day 1 and day 6, whereas animals fed ad lib did not exhibit a change in BW over days. Additionally, ad lib fed animals had higher BW than RF animals on all snack days,  $F(1, 38) = 30.45, p < 0.001, \eta_p^2 = 0.44$ .

Female animals offered 45% HFD showed a similar pattern of BW changes during the snacking period. Ad lib fed females offered 45% HFD had significantly higher BW on all days,  $F(1, 59) = 16.76, p < 0.001, \eta_p^2 = 0.22$ . A significant day by feeding schedule interaction,  $F(4, 236) = 6.64, p < 0.001, \eta_p^2 = 0.10$ , is characterized by a significant change in BW between day 1 and day 6 for RF females that were offered 45% HFD snacks while ad lib females offered 45% HFD did not exhibit any change in BW across days. As was expected, because male mice are generally larger than females, male RF mice offered 45% HFD snacks weighed more than their females counterparts in each treatment condition,  $F(1, 47) = 15.63, p < 0.001, \eta_p^2 = 0.25$ .

As shown in Fig. 2a, ad lib fed male mice receiving 45% HFD snacks consumed significantly fewer kcals/ g-bw during the snack than did RF animals on all days,  $F(1, 23) = 104.19, p < 0.001, \eta_p^2 = 0.82$ . All male animals offered the 45% HFD snack, regardless of restriction status, ate significantly more kcals/ g-bw over days of exposure to the 30 min access,  $F(5, 115) = 38.09, p < 0.001, \eta_p^2 = 0.62$ . The female pattern of

intake (Fig. 2b) was similar to males in that females offered 45% HFD snacks and not restricted calorically (ad lib animals) ate significantly less during the snacks (kcal/ g-bw) than did RF female animals,  $F(1, 16) = 511.92$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.97$ , and, regardless of feeding schedule, intake was significantly increased from day 1 to day 6,  $F(5, 80) = 29.80$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.65$ .

As noted in the previous section, female RF animals offered 45% HFD consumed more kcal/ g-bw than male RF animals. Similar comparisons for ad lib fed animals revealed that although both males and females exhibited increases in 45% HFD snack intake over days,  $F(5, 150) = 50.47$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.63$ , ad lib fed males and females offered 45% HFD consumed similar amounts of the HFD.

## *Experiment 2*

Two-way Repeated Measures ANOVAs were performed for BW and snack intake with day x restriction schedule (RF or ad lib) for female animals offered the 84% HFD as a snack.

84% HFD snacks – RF versus ad lib fed animals (females). RF animals increased in BW each day whereas ad lib fed animals exhibited no change in BW across days, revealed by a significant day by feeding schedule interaction,  $F(5, 180) = 4.911$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.12$ . Post hoc analyses revealed that a significant change in BW occurred for RF animals each day but not for ad lib fed animals. (See Table 3)

As shown in Fig. 3, RF animals exhibited a significant increase in snack intake (kcal/ g-bw), whereas ad lib fed animals exhibited no significant change in 84% HFD

snack intake across days as revealed by a significant day by feeding schedule interaction,

$F(5, 130) = 5.39, p < 0.001, \eta_p^2 = 0.17$ . Post hoc tests revealed that RF animals had significant increases in intake (compared to day 1) for days 4 through 6. Additionally, RF animals ate significantly more kcals/ g-bw during the snack than ad lib fed animals on all days,  $F(1, 26) = 38.99, p < 0.001, \eta_p^2 = 0.60$ .

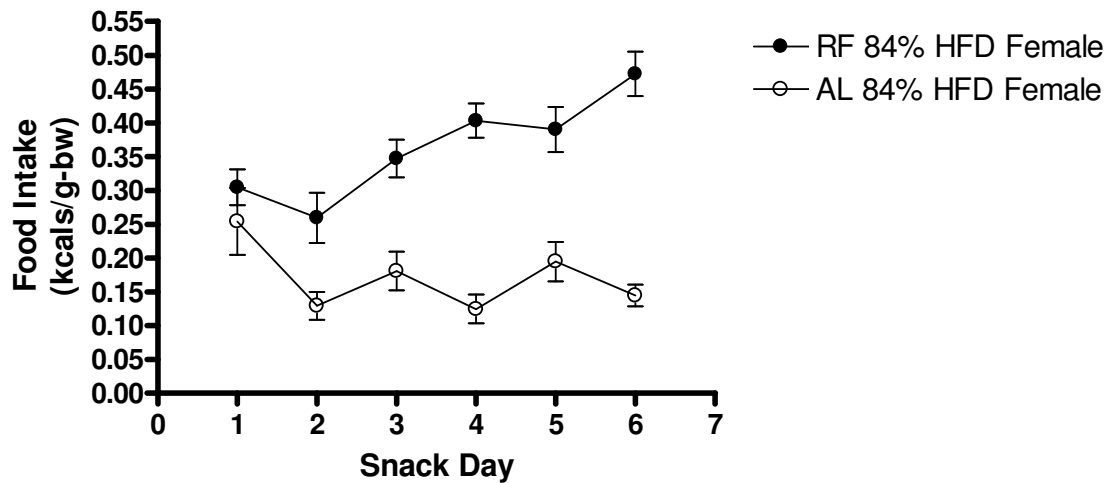


Fig. 3. Snack Intake for 84% HFD Snacks. Intake (kcals/g-bw) for female mice offered an 84% HFD snack on Monday, Wednesday, and Friday afternoons. RF = animals maintained on a restricted feeding schedule of 2 h/ d chow access. AL = animals maintained with ad lib chow access.



Circadian recordings for females offered 84% HFD snacks. Fig. 4 represents the timeline of circadian activity and highlights the 1 h increments of interest in circadian analyses. As seen in Fig. 5a, RF animals exhibited higher activity levels than ad lib fed animals during the hour preceding the 30 min afternoon snack as revealed by a significant main effect of feeding schedule,  $F(1, 13) = 8.58$ ,  $p = 0.01$ ,  $\eta_p^2 = 0.40$ . Additionally, planned comparisons for day 1 versus day 6 revealed that RF animals had

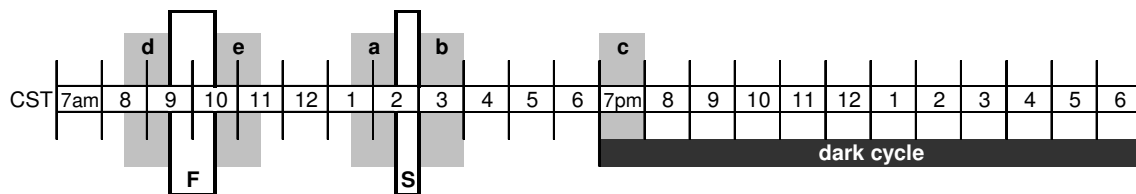
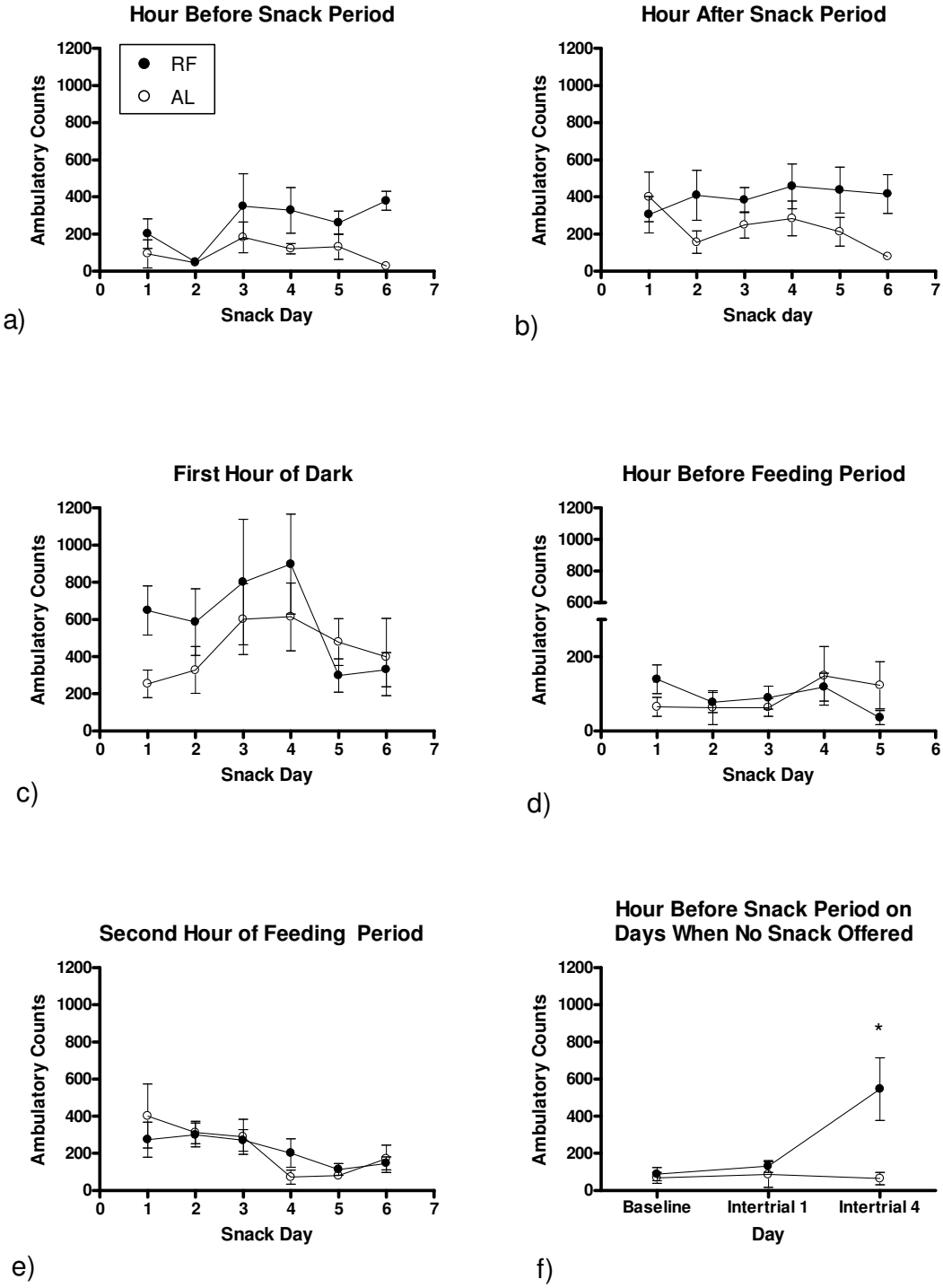


Fig. 4. Timeline for Circadian Measures. Time periods of interest for each snack day are represented. Vertical gray bars labeled **a** through **e** represent the 1 h periods used in analyses of circadian activity and correspond to subparts **a** through **e** of Figure 5. Bars labeled **F** and **S** indicate test periods in which an experimenter was present in the room (**F** = morning feeding period; **S** = 30 min snack).

significantly higher activity counts on day 6 than they did on day 1 of snacks, suggesting that RF animals acquired increased anticipatory response for the snack period compared to the ad lib fed animals. This pattern of increased activity was also apparent for the hour immediately following the snack (Fig. 5b) in that the RF animals had higher

Fig. 5. Ambulatory Counts for Circadian Measures. Circadian activity counts summed into 1 h increments for females offered 84% HFD snacks while being maintained on a restricted feeding schedule (closed circles) or ad lib chow access (open circles). a) 1 h sum of activity counts during the hour preceding the afternoon snack (1:30 – 2:30 pm CST) b) 1 h sum of activity counts during the hour following the removal of any remaining HFD snack (3:00 – 4:00 pm CST). c) Summed activity counts for the first h of the dark cycle (7:00 – 8:00 pm CST). d) Sum of activity counts for the 1 h period preceding the morning feeding period and BW measure (8:30 – 9:30 am CST). e) Sum of the second h of the 2 h feeding period (10:30 – 11:30 am CST). f) Sum of ambulatory counts during the pre-snack anticipatory period on the days when HFD snack was not provided. Baseline recording occurred on the day preceding the onset of snack sessions, and the intertrial days are days in between snack sessions where intertrial 1 followed the first snack day and intertrial 4 represents data collected after 4 snack sessions. \* $p < 0.05$

(See associated Fig. 4 for timeline of circadian intervals.)



activity counts than ad lib fed animals,  $F(1, 11) = 5.51$ ,  $p = 0.04$ ,  $\eta_p^2 = 0.33$ . No increases in activity during the post-snack period were seen across days (main effect of day, ns). However, planned comparisons showed that the ad lib fed animals had a statistically marginal ( $p = .051$ ) decrease in activity during the hour after the snack by day 6 of snacking. Together this suggests a shift towards increased activity at snack time for RF animals but not for ad lib fed animals.

The first hour of the dark cycle is typically the most active and contains the highest level of food intake for animals that are allowed to follow their normal circadian patterns. Planned comparisons of the first hour of the dark cycle (Fig. 5c) revealed higher activity counts for RF animals over ad lib fed animals on day 1 of snacking, however this difference was only marginally statistically significant ( $p = 0.054$ ) (ANOVA, ns). This marginal difference did not persist past the first day of snacking (see Fig. 5c). For RF animals, a significant decrease on activity counts was apparent between day 1 and day 6 of snack tests such that, by day 6, activity counts during the first hour of dark were similar to the daytime activity counts of the other time points addressed here (See Fig. 5).

As seen in Fig. 5d, the anticipatory activity that occurred during the hour preceding the scheduled 2 h feeding period was not significantly different between RF and ad lib fed animals. However, planned comparisons between these groups on day 1 of snacking revealed that RF animals had higher activity levels than ad lib fed animals on day 1 only ( $p < 0.05$ ). Because the experimenter was in the room during the first hour of the feeding period to provide diet access and measure BW, the 1 h period after

the experimenter had left the room was used in analyses of activity during the feeding period (2<sup>nd</sup> hour of the 2 h feeding period, see Fig. 4). Repeated Measures ANOVA revealed a significant effect of day,  $F(5, 60) = 3.82$ ,  $p = 0.005$ ,  $\eta_p^2 = 0.24$ , with both RF and ad lib animals exhibiting a decrease in activity during the second h of the feeding period over snack days (Fig. 5e). This decrease further supports a shift in activity to snack time as it coincides with the RF increase in pre-snack activity and the RF decrease in activity in the first hour of dark (Fig. 5a, 5c, and 5e).

Additionally, the increases in activity during the presnack hour persisted during intertrial days. Since limited access to the HFD was provided with a minimum of 1 non-access day between snack sessions (intertrial days), Fig. 5f shows the activity counts during the baseline (before snacks began), intertrial 1 (after only 1 HFD access), and intertrial 4 (after 4 HFD access periods). Repeated Measures ANOVA for these intertrial and baseline days revealed a marginal interaction effect of feeding schedule by day,  $F(2, 30) = 3.11$ ,  $p = 0.059$ ,  $\eta_p^2 = 0.17$ , and a main effect of feeding schedule,  $F(1, 15) = 5.40$ ,  $p = 0.03$ ,  $\eta_p^2 = 0.2$ , such that animals maintained on the RF schedule exhibited a significant increase in daily activity after 4 HFD access periods regardless of whether HFD snack was offered on the day of recording. This suggests that the increased arousal/activity in anticipation of the HFD snack was not dependent on learning of the schedule of access. As seen in pre-snack activity on HFD snack days, ad lib fed animals showed no changes in pre-snack activity on intertrial days.

Rebound feeding after 84% HFD snacks. Analysis of BW data for the rebound period was conducted as time (pre-rebound, week 1, and week2) x diet (45% or 84% HFD) x previous feeding schedule (RF or ad lib) Repeated Measures ANOVA. This analysis yielded a significant schedule by diet interaction,  $F(1, 33) = 86.52, p = 0.02, \eta_p^2 = 0.14$ , with the ad lib animals offered 45% HFD exhibiting no change in BW during rebound compared to pre-rebound. This analysis also produced a time by schedule interaction,  $F(2, 66) = 9.16, p < 0.001, \eta_p^2 = 0.22$ , with post hoc tests revealing that the RF animals gained weight over time regardless of diet offered during rebound. Additionally, a time by diet interaction,  $F(2, 66) = 6.29, p = 0.003, \eta_p^2 = 0.16$ , was also revealed with post hoc tests showing that all animals offered 84% HFD gained weight during rebound compared to pre-rebound. Finally, a main effect for week,  $F(2, 66) = 47.16, p < 0.001, \eta_p^2 = 0.59$  (Fig. 6a), was yielded in this analysis such that, taken together, BW changed over weeks.

As represented in Fig. 6b, Repeated Measures ANOVA of the average daily intake (kcal/ g- bw) for each week of rebound feeding yielded a significant three-way interaction (week x feeding schedule x rebound diet,  $F(1, 34) = 17.5, p < 0.001, \eta_p^2 = 0.34$ ). Post hoc tests revealed that RF animals offered the 84% HFD during rebound ate more daily kcal/ g-bw than did RF animals offered 45% HFD during rebound feeding and exhibited a significant decrease in intake from week 1 to week 2 of rebound feeding.

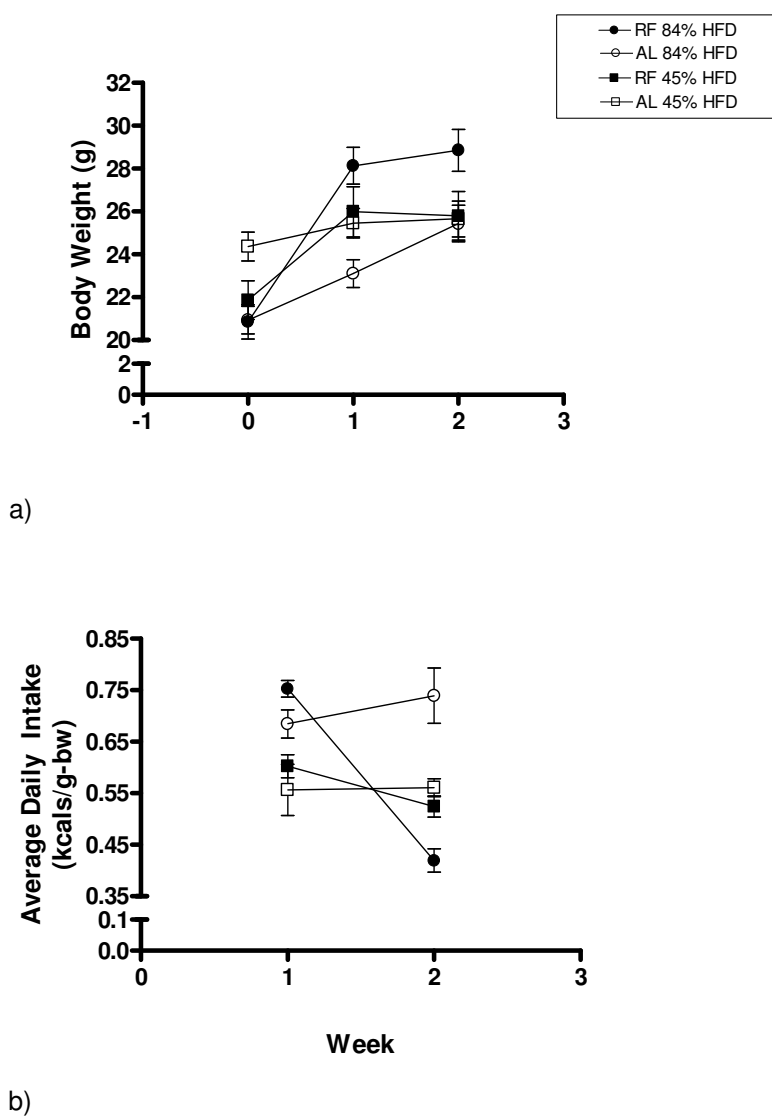


Fig. 6. Rebound Experiment BW and Food Intake. Body weight (a) and intake (b) for the 2 week rebound period in which all animals received ad lib access to either 45% or 84% HFD after receiving 6 snack sessions with the 84% HFD. Measures are represented mean (+/- sem) of the daily average for each animal during each week of rebound. a) average BW for each week of rebound where time = 0 indicates the pre-rebound period (last day of snack tests) b) daily average intake (kcal/g-bw) for each week of rebound RF = animals previously maintained on restricted feeding schedule. AL = animals previously maintained on ad lib chow.

Ad lib fed animals offered 84% HFD ate more daily kcals/ g-bw during week 2 than RF animals offered the same rebound diet. Ad lib fed animals offered 84% HFD during rebound exhibited higher intakes (kcals/ g-bw) than did ad lib fed animals offered 45% HFD during rebound feeding. (See Fig. 6b).

The analysis of intake also yielded a significant week by group interaction,  $F(1, 34) = 41.60$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.55$ , in that the RF groups had higher intakes than ad lib animals during week 1 but this pattern did not persist to week 2 where RF intakes dropped below the ad lib intakes. Additionally, a significant week by rebound diet interaction,  $F(1, 34) = 7.85$ ,  $p = 0.008$ ,  $\eta_p^2 = 0.19$ , revealed that animals receiving 84% HFD had higher intakes during week 1, but not during week 2. A significant group by rebound diet interaction,  $F(1, 34) = 6.53$ ,  $p = 0.02$ ,  $\eta_p^2 = 0.16$ , was also achieved, however, this interaction and the week by rebound diet interaction effects are likely driven by the dramatic decrease in intakes for the RF animals offered 84% HFD.

Additionally, the main effects of feeding schedule,  $F(1, 34) = 5.63$ ,  $p = 0.02$ ,  $\eta_p^2 = 0.14$ , rebound diet,  $F(1, 34) = 11.36$ ,  $p = 0.001$ ,  $\eta_p^2 = 0.26$ , and week,  $F(1, 34) = 23.36$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.41$ , were difficult to interpret in the context of the significant interactions generated in this analysis.

Finally, due to the somewhat complicated nature of the interaction effects yielded in analysis of BW during rebound, the additional calculated variable of percent change in BW between the last snack day and week 2 of rebound was calculated as: (week 2



BW – pre-rebound BW) / week 2 BW (\*100). A diet (45% or 84% HFD) by previous feeding schedule (RF or ad lib) ANOVA revealed that RF animals exhibited higher percent change in BW than ad lib animals,  $F(1, 34) = 38.09$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.53$ , and that animals offered 84% HFD had a greater percent change in BW during rebound than animals offered 45% HFD,  $F(1, 34) = 59.62$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.63$ . (See Fig. 7)

*Diet Comparisons (Exp 1 versus Exp 2 Snacking)*

BW, latency, and intake data for all female animals from snacking phases of Experiment 1 and Experiment 2 were analyzed in order to make comparisons about the varied nutritional content of the 45% and 84% HFD. These experiments were not run concurrently, however, every effort was made to ensure identical procedures were implemented for both sets of animals throughout each experiment.

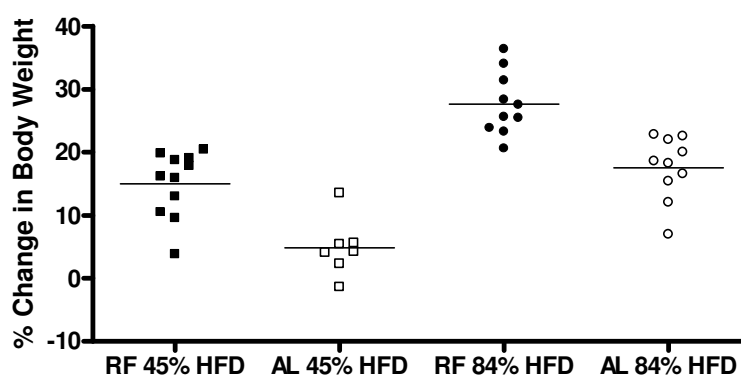


Fig. 7. Percent Change in BW During Rebound Feeding. Calculated data of percent change between the last snack day and the second week of rebound feeding. RF = maintained on restricted feeding during snacking; AL = maintained on ad lib chow during snacking.

Female RF animals offered the 84% HFD snacks exhibited a significant increase in BW starting on day 3 of snacking whereas animals offered the 45% HFD did not exhibit a significant increase in BW until day 5 as revealed by a significant day x diet interaction,  $F(5, 115) = 5.63$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.20$ . (See Table 3 for means +/- sem)

Three-way Repeated Measures ANOVA for intakes (kcal/g-bw) for snack day by feeding schedule (RF, ad lib) by diet (45%, 84% HFD) yielded a significant 3-way interaction effect,  $F(5, 210) = 2.57$ ,  $p = 0.03$ ,  $\eta_p^2 = 0.06$ , and this effect (Fig. 8a) is most likely driven by the fact that all groups showed a significant increase in intakes over days except for the ad lib animals offered 84% HFD, which exhibited decreased intake over days. Furthermore, the effect size for the 3-way interaction is small revealing that this interaction effect did not account for much of the overall variance in this analysis. For animals maintained on the RF schedule, no differences existed between intakes for 45% or 84% HFD snacks; however, these animals exhibited a significant increase in kcal/g-bw consumed across days (Fig. 8a), while ad lib fed animals exhibited no such increase, evidenced by the significant day by feeding schedule interaction,  $F(5, 210) = 4.27$ ,  $p = 0.001$ ,  $\eta_p^2 = 0.09$ . Animals maintained on ad lib chow, but not RF animals, ate significantly more kcal/g-bw on day 1 when offered the 84% HFD than when offered the 45% HFD, but this difference did not persist past day 1, diet by feeding schedule interaction:  $F(1, 42) = 5.75$ ,  $p = 0.02$ ,  $\eta_p^2 = 0.12$ . For both diets (45% HFD or 84% HFD), female animals subjected to the RF schedule consumed significantly more kcal/g-

g-bw than animals maintained on ad lib chow throughout snacking, main effect of feeding schedule:  $F(1, 42) = 138.79$ ,  $p < 0.001$ ,  $\eta_p^2 = 0.77$ , with the exception of day 1 for RF animals offered 84% HFD snacks (Fig. 8a).

As seen in Fig. 8b, the animals receiving 45% HFD snacks had higher latencies than those offered the 84% HFD snacks on all snack days,  $F(1, 22) = 5.87$ ,  $p = 0.02$ ,  $\eta_p^2 = 0.21$ , possibly suggesting increased motivation to eat the 84% HFD.

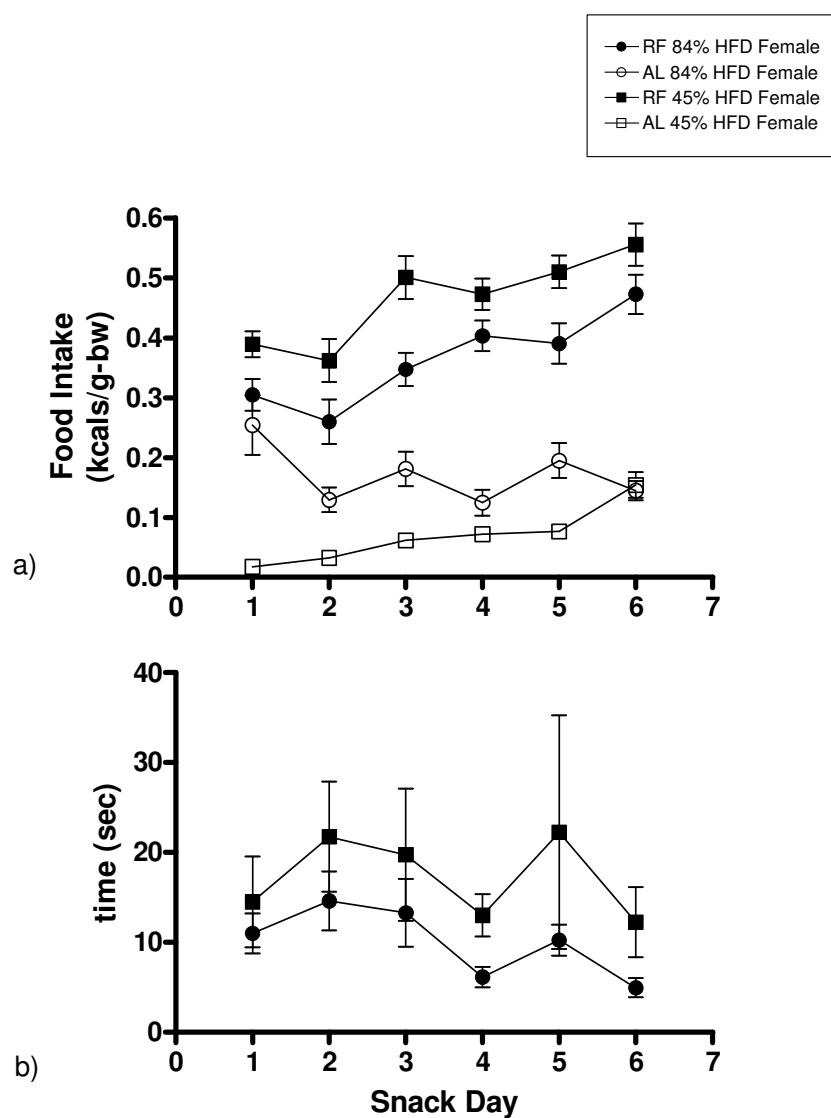


Fig. 8. Intake and Latency Comparisons for 45% and 84% HFD Snacks. Snack data from female animals in both Experiment 1 and Experiment 2a are represented here. a) Intake in kcal/g-bw for each snack day. Day 6 for animals receiving the 45% HFD is missing data due to lost BW recordings such that kcal/g-bw could not be calculated. b) Latency to begin eating the HFD snack. RF = animals maintained on restricted feeding schedule. AL = animals maintained on ad lib chow.

## SUMMARY AND CONCLUSIONS

A key finding of this investigation is that the limited-access model can be replicated in mice as evidenced by increases in intake of 45% HFD over successive exposures to the diet, and this binge-effect is not dependent on restriction. This finding is further supported by the fact that animals offered limited access to chow, but not HFD, while on a restricted feeding schedule do not show similar increases over days but consumed a reasonably regulated amount of chow during the snack periods each day. These results are congruent with results reported by Corwin and colleagues [34, 39] in that caloric restriction was not necessary to produce increasing intakes of HFD over multiple access periods. The results of the current investigation provide added information about this model in that animals maintained on a RF schedule, while having higher baseline HFD intakes, did not show a greater magnitude of binge-behavior from day 1 to day 6 access when compared to the ad lib controls. However, the higher baseline HFD intakes for animals maintained on the RF schedule may more readily model the excessive binges that are prevalent in binge-eating pathology where daily caloric restriction is the norm. More specifically, the limited-access model without caloric restriction might most closely model bingeing that accompanies restricting access to palatable foods only, whereas, the current investigation shows that caloric restriction (dieting) in combination with limited-access to palatable food produces a greater short-term 'binge'-effect without necessarily increasing the magnitude of the change in intake over prolonged periods of HFD limitation. These results might be explained by an additive effect in that reward mechanisms induce bingeing in response to limited access

to a palatable diet, and when homeostatic need is added, the magnitude of the binge is increased.

Corwin's model [34, 39] of limited-access bingeing used vegetable shortening as the palatable offering, and the current results support the use of a nutritionally complete diet (45% HFD) to achieve the same increases in intake over days. However, this investigation did not find the expected increase in HFD intake for unrestricted animals when the diet offered was the 84% HFD (ketogenic), which more closely approximates vegetable shortening. The unrestricted animals showed a greater initial intake (snack day 1) of the 84% HFD than similar animals offered the 45% HFD, but unrestricted animals offered the 84% HFD did not show successive increases in intake over multiple exposures. This cannot be explained by a ceiling effect in that RF animals consumed many more kcals/ g-bw than did ad lib fed animals, so time and stomach volume limitations cannot account for this lack of 'binge'. However, it might be concluded that the increased palatability of the 84% HFD produced a ceiling effect on the rewarding properties of the diet such that ad lib fed animals, eating only for reward and not nutritional need, maintained reasonably steady intake of the 84% HFD in support of the reward value gained by doing so. This is in direct contrast to Corwin's limited access model where ad lib fed animals ate increasing amounts of non-nutritive vegetable shortening. The current results do show increases in 84% HFD intakes when the animals were on the RF schedule making it possible to conclude that the combination of restriction and HFD access reliably produces bingeing in mice. However, the inconsistent findings in ad lib fed mice offered the 45% or 84% HFD make it difficult to

ascertain why the limited-access replication was not absolute for mice (versus rats in Corwin model [34, 39]).

The disparity between diets offered in the limited access model is also evidenced in comparisons between the two experiments. For animals maintained on the RF schedule, intakes of the 45% HFD were higher on all days than were the intakes of 84% HFD. In contrast, ad lib fed animals consumed less when offered the 45% HFD than similar animals offered the 84% HFD. As seen in Fig. 7, the differences in intakes produced by the maintenance diet (RF versus ad lib) are lesser in magnitude when the animals were offered 84% HFD snacks than when they were offered 45% HFD snacks. Again, this suggests the possibility that the convergence of homeostatic mechanisms and reward mechanisms may produce an additive effect on behavior. Specifically, the 45% HFD was a nutritionally complete diet (see Table 2) such that the drive for binge-eating when both homeostatic need and reward properties (fat content) were present was high. In contrast, the 84% HFD was ketogenic (lacking in carbohydrates) and lower in other nutritional elements, due to the high fat content, which may have reduced the drive for consumption to a purely reward-mediated process for all animals. This idea is also supported by the lower intakes of 45% HFD for ad lib fed animals in that the potential reward component (reduced fat = reduced palatability) was not as strong as the reward component produced by the 84% HFD. Therefore, it is plausible to conclude that the results of this investigation support the dissociation between the reward components and homeostatic components of binge-behavior in response to varying diet features, although more research is needed on this point.

The findings reported here support the notion that females show a greater magnitude of binge than males when they are restricted. However, when animals in this study were not restricted (maintained on ad lib chow), there were no differences between males and females. When intakes were corrected for BW difference, RF females had higher intakes (kcal/g-bw) than RF males in the 30 min snack sessions of HFD. As mentioned earlier, Hudson [5] reports BN in the USA to be 1.5% of women and 0.5% of men, while BED is reported to affect 3.5% of women and 2.0% of men. Therefore, the results of this investigation may lend support for an increased susceptibility of females to binge-eating. Considering that BN is more likely to be accompanied by caloric restriction, the RF animals in the current investigation show that females restricted calorically binge with greater magnitude than males when palatable food is offered. Furthermore, the prevalence of BED is less disparate between the sexes and less likely to be accompanied by caloric restriction making the results reported here also in support of the human condition in that no sex differences in binge magnitude existed for animals that were not calorically restricted.

Inspection of the latencies to begin eating during the limited-access snack periods supports the idea that the 84% HFD may have increased reward value compared to the 45% HFD. Specifically, no differences were seen in latency between animals offered the 45% HFD and chow snack. However, animals offered the 84% HFD had decreased latency to begin eating during the snack period compared to mice offered the 45% HFD. This decrease in latency might be interpreted as increased motivation to eat the palatable snack food. As is discussed below, the restricted mice offered the 84% HFD showed



alterations in their food-associated activity patterns. Therefore, it is possible that the latency measure is just an extension of the evidence in support of increased arousal at snack time.

The recordings of circadian ambulatory counts in this study clearly show that animals subjected to an RF schedule and offered limited-access to 84% HFD show alterations in their feeding-related activity patterns that are not seen in ad lib fed animals offered the same limited-access HFD. Specifically, RF animals exhibited increases in anticipatory activity during the hour preceding the snack after multiple exposures to the HFD snack and maintained a high level of activity during the hour after the snack period. In contrast, ad lib fed animals showed no anticipatory increases in activity in the hour before the snack and exhibited a decrease in activity after the snack period over multiple exposures to the HFD snack. Furthermore, the shift in activity towards the snack period for RF animals is further evidenced by a decrease in activity during the first hour of the dark period and in the hour preceding the morning 2 h chow access. This supports the notion that maintenance on the RF feeding schedule, resulting in caloric restriction (dieting), produced a shift in arousal associated with access to the 84% HFD, while access to the 84% HFD without restriction produced no such shift. However, a limitation to interpretation of the activity analyses here is the lack of similar recordings for animals offered the 45% HFD. In light of the binge-eating results discussed above, it is plausible that the inspection of the differences in circadian activity in response to different diet features (i.e., fat content, nutritional content) may have yielded a clearer

picture of the dissociation between homeostatic shifts in activity and reward based changes in activity.

Considering the lack of ‘binge’-effect (increasing intakes over days) in ad lib fed animals offered limited-access to the 84% HFD, the lack of change in circadian activity patterns for these animals may support the idea that circadian fluctuations in activity are more readily produced by the homeostatic mechanisms that influence feeding. Previous research has shown that various restriction regimens produce changes in circadian rhythmicity [44, 47, 48, 49] supporting the notion that the induction of homeostatic need causes changes in arousal and energy expenditure. Ad lib access to palatable foods without any form of caloric restriction has also been shown to alter activity patterns [50, 51] with more variability in that both pattern shifts and general decreases are reported. However, none of the past research considered the combination of restriction and palatable diet offerings. The current investigation supports the role of homeostatic need in altering food-associated arousal, but discredits the idea that access to palatable diet alone can produce such alterations. As noted above, the limitations of the current investigation limit the interpretation of these results, and further study is needed to fully elucidate the potential influences of bingeing on food-associated arousal.

The final aim of this investigation was to inspect the rebound feeding of animals offered a familiar HFD (experienced during limited access) or an unfamiliar HFD. As evidenced by both the actual BW and the percent change in BW between the last snack day and week 2 of rebound, animals maintained on RF during limited-access to the 84% HFD had higher BW and gained more weight during rebound regardless of which diet

was offered during rebound. This is indicative of the influence that dietary restriction has been shown to have on ad lib eating of palatable foods in humans [2, 6] in that restriction leads to increased intake and increases the likelihood of binge-eating behavior. This is further evidenced by the fact that RF animals offered the binge-associated 84% HFD showed the largest percent change in BW and highest actual BW during week 2 of rebound.

During week 1 of ad lib access to the previously limited-access 84% HFD, animals with a history of RF and higher intakes than all other groups and exhibited a marked decline in intake during week 2. Together, this may indicate that a history of bingeing on the 84% HFD carried over to ad lib access such that overeating occurred during week 1 when this diet was offered ad lib. However, to make better conclusions on this point, animals receiving limited-access to 45% HFD and 45% HFD during rebound would need to be inspected. In the context of the binge-eating differences between the 2 diet types outlined above, it may also be concluded that the diet features in combination with a history of bingeing may exert differential influences on rebound behavior, and a more thorough investigation of the potential combined influences is needed to make a full interpretation of the carry-over effects of binge-related foods.

It is also important to note that, because the 2 diets (45% HFD and 84% HFD) provided during rebound feeding were nutritionally different, the increases in intake for the groups receiving 84% HFD may be confounded by the lack of carbohydrates and decreased nutritional elements in this diet. Therefore, an alternate explanation for the rebound feeding results presented here is that animals offered the 84% HFD had

increased intakes compared to the 45% HFD groups due to the need to compensate for decreased nutritional value of the 84% HFD. However, this explanation is not fully supported by the data in that a marked decrease in 84% HFD consumption was recorded for RF animals during week 2 of rebound feeding. Evidence exists for alterations in immune function [54] and gene expression [55] in protein deficient diets, but no such evidence could be found for reduced or altered functions produced by a diet lacking in carbohydrates. Therefore, it is possible that the nutritional content might not play as big a role as palatability in the intake and BW changes seen during rebound feeding.

In summary, the findings of the current investigation support the use of the limited access model in mice while raising many more questions about the potential interplay between homeostatic need and reward in binge eating models. A drawback to binge-eating animal models that employ R/R with stress is that, while accurately modeling the potential stress-triggers of bingeing, they add the potential confound of a physical stressor to induce bingeing and do not rely solely on the reward properties of palatable food to induce bingeing. The limited-access paradigm corrects for this confound but does not replicate the human condition of caloric restriction that is seen in many binge-pathologies. Therefore, a key contribution of the current investigation is to add a 'dieting' component to the limited-access model thereby allowing the inspection of the interplay between homeostatic drive and reward-based bingeing. The addition of this aspect of the model then creates a paradigm under which the hormonal, genetic, and neurochemical modulators of human binge-eating pathology can be more clearly elucidated in the context of energy needs and reward.

Because human binge pathology is closely linked to a perceived restriction of palatable foods and often an actual restriction on caloric intake in order to offset binge behavior, the relationship between the psychological perception of reward-deficit and the actual energy deficits has proved difficult to model in animals. The results of this investigation, by adding a ‘dieting’ component to the limited-access model, may have produced more questions than answers about the ‘need’ versus ‘want’ issues in bingeing. However, the results reported here clearly support a future line of questioning which inspects the coordinated behavioral controls that may be exerted by both limiting access to palatable foods (reward) and limiting nutritional intake. It is also important to note that, if the interplay between homeostatic need and reward systems proves to be an important feature of binge-eating, then this may elucidate clinical implications concerning the need for nutritional satiation to aid in the control of binges. Furthermore, the extended limited-access model reported here may also provide the means to investigate binge triggers by the simple addition of a stress component in order to investigate these triggers in the context of the combined physiological (homeostatic) and psychological (reward) process that may modulate binge-eating behavior. In short this model, with the use of multiple diet features, allows for further investigation of the dissociation between homeostatic and reward based bingeing that are both relevant to human pathology.

A major drawback in the modeling of binge-behavior is that there is no clear definition of ‘binge’. Corwin’s group [34, 39] reports bingeing with 51% of daily intake occurring during the binge episode, while R/R models [28, 29, 30] define ‘binge’ only in

comparison to the intake of control animals. Therefore, an important aspect for the improvement of animal models is to more clearly define what constitutes a pathological-type binge-episode and what is merely overeating of palatable foods. This distinction is important in that most humans will experience overeating of palatable foods but pathological bingeing is much more extreme. Furthermore, clinical definitions of binge-pathology are based on the disruptive aspect to the extreme binges [1] and, as such, represent a situation where the potential physiological mechanisms controlling these pathologies are likely to be different from those that regulate normal reward-based overeating of palatable foods. Therefore, a major limitation of all binge-models reported in the literature is that this distinction between pathology and normal human experience cannot be made.

In conclusion, the findings of this investigation clearly support the hypothesis that homeostatic need can alter binge behavior, arousal (activity), and subsequent access to a previously restricted palatable diet. Differences in diet features (nutritional content), homeostatic energy status, and reward may exert singular influences on binge behavior, and the complex relationship between each of these elements may also produce additive effects upon the complex behaviors associated with bingeing.

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